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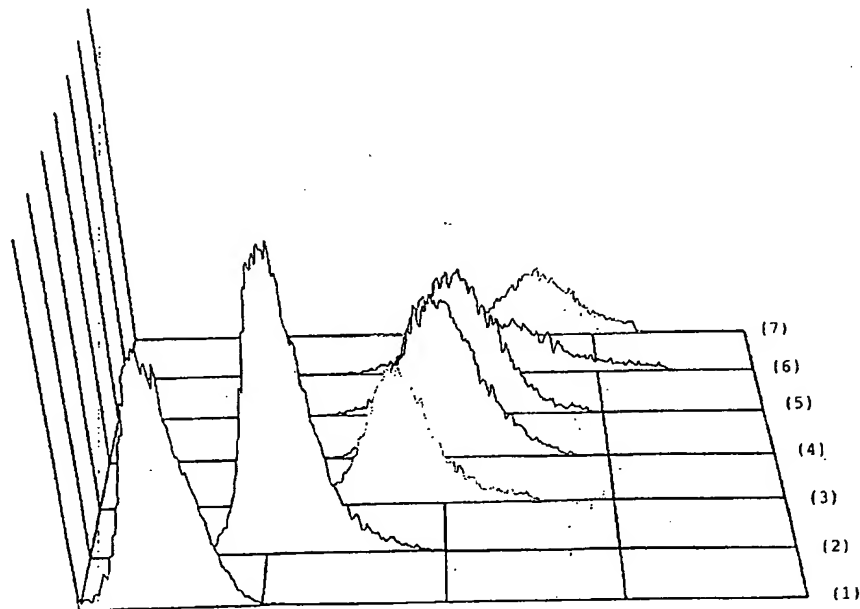
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(54) Title: A VACCINE WHICH COMPRISES AT LEAST ONE ANTIGEN AND A CATHELIDIDIN DERIVED ANTIMICROBIAL PEPTIDE OR A DERIVATIVE THEREOF



(57) Abstract: Described is a vaccine which comprises at least one antigen and at least one cathelicidin derived antimicrobial peptide or a derivative thereof as well as the use of a cathelicidin derived antimicrobial peptide or a derivative thereof for the preparation of an adjuvant for enhancing the immune response to at least one antigen.

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**A vaccine which comprises at least one antigen and a  
cathelicidin derived antimicrobial peptide or a derivative  
thereof**

The present invention relates to vaccines comprising at least one antigen and an immunostimulating substance.

Host protection from invading pathogens involves cellular and humoral effectors and results from the concerted action of both non-adaptive (innate) and adaptive (acquired) immunity. The latter is based on specific immunological recognition mediated by receptors, is a recent acquisition of the immune system, and is present only in vertebrates. The former evolved before the development of adaptive immunity, consisting of a variety of cells and molecules distributed throughout the organism with the task of keeping potential pathogens under control (Boman 2000), (Zanetti, Gennaro et al. 1997).

B and T lymphocytes are the mediators of acquired antigen specific adaptive immunity, including the development of immunological memory, which is the main goal of creating a successful vaccine (Schijns 2000). Antigen presenting cells (APCs) are highly specialized cells that can process antigens and display their processed fragments on the cell surface together with molecules required for lymphocyte activation. This means that APCs are very important for the initiation of specific immune reactions. The main APCs for T lymphocyte activation are dendritic cells (DCs), macrophages, and B cells, whereas the main APCs for B cells are follicular dendritic cells. In general DCs are the most powerful APCs in terms of initiation of immune responses stimulating quiescent naive and memory B and T lymphocytes (Banchereau, Briere et al. 2000).

The natural task of APCs in the periphery (e.g. DCs or Langerhans cells) is to capture and process antigens, thereby being activated they start to express lymphocyte co-stimulatory molecules, migrate to lymphoid organs, secrete cytokines and present antigens to different populations of lymphocytes, initiating antigen specific immune responses. They not only activate lymphocytes, under certain circumstances, they also

tolerize T cells to antigens (Banchereau and Steinman 1998).

Antigen recognition by T lymphocytes is major histocompatibility complex (MHC) restricted. A given T lymphocyte will recognize an antigen only when the peptide is bound to a particular MHC molecule. In general, T lymphocytes are stimulated only in the presence of self MHC molecules, and antigen is recognized only as peptides bound to self MHC molecules. MHC restriction defines T lymphocyte specificity in terms of the antigen recognized and in terms of the MHC molecule that binds its peptide fragment.

Intracellular and extracellular antigens present quite different challenges to the immune system, both in terms of recognition and of appropriate response. Presentation of antigens to T cells is mediated by two distinct classes of molecules - MHC class I (MHC-I) and MHC class II (MHC-II), which utilize distinct antigen processing pathways. Mainly one could distinguish between two major antigen processing pathways that have evolved. Peptides derived from intracellular antigens are presented to CD8<sup>+</sup> T cells by MHC class I molecules, which are expressed on virtually all cells, while extracellular antigen-derived peptides are presented to CD4<sup>+</sup> T cells by MHC class II molecules (Monaco 1992), (Harding 1995). However there are certain exceptions to this dichotomy. Several studies have shown that peptides generated from endocytosed particulate or soluble proteins are presented on MHC-I molecules in macrophages as well as in dendritic cells (Harding 1996), (Brossart and Bevan 1997). Therefore APCs like dendritic cells sitting in the periphery, exerting high potency to capture and process extracellular antigens and presenting them on MHC-I molecules to T lymphocytes are interesting targets in pulsing them extracellularly with antigens in vitro and in vivo.

The important and unique role of APCs, including stimulating activity on different types of leukocytes, is reflecting their central position as targets for appropriate strategies in developing successful vaccines. Theoretically one way to do so is to enhance or stimulate their natural task, the uptake of antigen(s). Once pulsed with the appropriate antigens the vaccine is directed against, APCs should start to process the

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taken up antigen(s), thereby being activated, expressing lymphocyte co-stimulatory molecules, migrating to lymphoid organs, secreting cytokines and presenting antigens to different populations of lymphocytes thereby initiating immune responses. Activated T cells generally secrete a number of effector cytokines in a highly regulated fashion, including interleukin 2 (IL-2), interferon- $\gamma$  (IFN- $\gamma$ ), tumor necrosis factor alpha (TNF- $\alpha$ ), IL-4, IL-5 and IL-10. The functional detection of cytotoxic T lymphocyte responses to specific antigens (e.g. tumor antigens, in general antigens administered in a vaccine) is commonly monitored by an ELISpot assay (enzyme-linked immunospot assay), a technique analyzing cytokine production at the single cell level. In the present invention an ELISpot assay for the cellular immunity promoting cytokine IFN- $\gamma$  was used to monitor successful peptide specific T cell activation.

It has previously been shown that polycations efficiently enhance the uptake of MHC class I-matched peptides into tumor cells, a peptide or protein pulsing process which was called "TRANSloading". (Buschle, Schmidt et al. 1997). Furthermore we have shown that polycations are able to "TRANSload" peptides or proteins into antigen presenting cells in vivo as well as in vitro (Buschle 1998). In addition, co-injection of a mixture of poly-L-arginine or poly-L-lysine together with an appropriate peptide as a vaccine protects animals from tumor growth in mouse models (Schmidt, Buschle et al. 1997). This chemically defined vaccine is able to induce a high number of antigen/peptide-specific T cells. That was shown to be at least partly attributable to an enhanced uptake of peptides into APCs mediated by the polycation (Buschle 1998) indicating that APCs when pulsed in vivo with antigens can induce T cell mediated immunity to the administered antigen.

As opposed to adaptive immunity, which is characterized by a highly specific but relatively slow response, innate immunity is based on effector mechanisms that are triggered by differences in the structure of microbial components relative to the host. These mechanisms can mount a fairly rapid initial response, which mainly leads to neutralization of the noxious agents. Reactions of innate immunity are the only defense strategy of

lower phyla and have been retained in vertebrates as a first line host defense before the adaptive immune system is mobilized.

In higher vertebrates the effector cells of innate immunity are neutrophils, macrophages and natural-killer (NK) cells and probably also dendritic cells (Mizukawa, Sugiyama et al. 1999), whereas the humoral components in this pathway are the complement cascade and a variety of different binding proteins (Boman 2000).

A rapid and effective component of innate immunity is the production of a large variety of microbicidal peptides with a length of between 12 and one hundred amino acid residues. Several hundred different antimicrobial peptides have been isolated from a variety of organisms, ranging from sponges, insects to animals and humans, which points to a widespread distribution of these molecules. Antimicrobial peptides are also produced by bacteria as antagonistic substances against competing organisms.

Main sources of antimicrobial peptides are granules of neutrophils and epithelial cells lining the respiratory, gastrointestinal and genitourinary tracts. In general they are found at those anatomical sites most exposed to microbial invasion, are secreted into internal body fluids or stored in cytoplasmic granules of professional phagocytes (neutrophils) (Ganz and Lehrer 1997), (Ganz and Lehrer 1998), (Lehrer and Ganz 1999), (Gudmundsson and Agerberth 1999).

The aim of the present invention is to provide an adjuvant/"carrier-peptide" which strongly enhances the immune response to a specific co-administered antigen.

A further object of the present invention is to provide an adjuvant/"carrier-peptide" known as a body-own molecule in animals, including humans, in particular mammals, and reducing the risk of mounting an immune response against the given adjuvant/"carrier-peptide" in animals including humans.

These objects are solved by a vaccine which comprises at least one antigen and at least one cathelicidin derived antimicrobial peptide or a derivative thereof.

Antimicrobial peptides can be grouped into five classes based on their known or expected 3D structure (Boman 2000).

The spectrum of organisms susceptible to the microbicidal activity of antimicrobial peptides is broad, including various bacteria (Gram positive & negative), protozoa, fungi and in some cases virus infected cells and tumor cells.

In general each species is equipped with a different array of these peptides that likely represents the outcome of an evolutionary selection dictated by the preferential association of a specific set of microbes with a given species.

All of the antimicrobial peptides known are produced by proteolytic processing from precursor molecules. In addition an important part of the biosynthesis of the effectors are the different forms of post-transcriptional modifications which are of importance to the final function like C-terminal amidation (e.g. indolicidin, PR-39, some beta-defensins (Bradbury and Smyth 1991), D-amino acid substitutions (Kreil 1997) or pyroglutamate blocking of the N-terminus (e.g. attacins and some beta-defensins).

One major family of cationic antimicrobial peptides (CAPs) in animals and humans are cathelicidins (Zanetti, Gennaro et al. 2000). Cathelicidins are derived from myeloid cells and have been identified in several mammalian species. So far, cathelicidins with masses ranging from 16-26 kDa were found to be expressed mainly in myeloid cells of human, mouse, cow, pig, horse, sheep, rabbit and rat. They are made as precursors, in which the highly identical N-terminal preprosequences are followed by highly varied C-terminal sequences that correspond to antimicrobial peptides after removal of the prosequence at specific cleavage sites (shown in Fig. 3, out of (Zanetti, Gennaro et al. 1997)).

The prosequences of all these congeners are highly homologous to the sequence of a protein named cathelin, first isolated from porcine leukocytes. Based on the common presence of this highly conserved cathelin-like domain, these precursors have been grouped into a family named cathelicidins.

The cathelin-like preproregion shows a high intra-species identity ranging from 75% for bovine, to complete identity for some of the porcine congeners. Four invariant cysteins clustered in the C-terminal region of the cathelin-like propiece are arranged to form two intramolecular disulfide bonds, imposing structural constraints to the molecule. The cathelin-like proregion shows limited homology to the cystatin family, proteins of known thiol protease inhibitory function. This is further supported by the moderate inhibitory effects exerted by several cathelicidins on the activity of the cysteine proteinase cathepsin L on which the acronym cathelin is based. Although a specific function for this prosequence has not been established, the evolutionary pressure exerted towards its conservation suggests it may play an important biological function, such as targeting of the antimicrobial peptides to the granules or aiding their correct proteolytic maturation.

The preproregion of cathelicidins is 128-143 amino acid residues long, including a putative 29-30 residue signal peptide and a propiece of 99-114 residues, while the C-terminal domain is 12-100 residues long. When these propeptides are secreted, they undergo limited proteolysis. In bovine and porcine neutrophils, cathelicidins are liberated by elastase-mediated cleavage (Cole, Shi et al. 2001), while the human cathelicidin hCAP-18 is processed extracellularly to the antimicrobial peptide LL-37 by proteinase 3 (Sorensen, Follin et al. 2001) indicating that the generation of active antimicrobial peptides from common proproteins occurs differently in related species.

Cathelicidins were first found in secondary granules of neutrophils (Gudmundsson, Agerberth et al. 1996), (Gudmundsson and Agerberth 1999). Thus, cathelicidins are released into inflammatory fluids where they are found at relatively high concentrations (Agerberth, Grunewald et al. 1999), (Gudmundsson



and Agerberth 1999). The only cathelicidin found in humans so far, the peptide LL-37 (hCAP-18/FALL-39) is expressed in neutrophil granules and is produced by bone marrow and testis (Cowland, Johnsen et al. 1995), (Sorensen, Arnljots et al. 1997). Additionally, LL-37 is constitutively expressed in squamous epithelia of mouth, tongue, esophagus, cervix, and vagina (Frohm Nilsson, Sandstedt et al. 1999), the lung epithelia (Bals, Wang et al. 1998) and the epithelium of the epididymis (Malm, Sorensen et al. 2000). Furthermore, high levels of LL-37 were found in the seminal plasma (Malm, Sorensen et al. 2000). Moreover, LL-37 is induced in keratinocytes of inflamed skin (Frohm, Agerberth et al. 1997), is found in high concentrations in the lipoprotein fraction of plasma (Sorensen, Bratt et al. 1999) and in bronchoalveolar lavage fluid (Agerberth, Grunewald et al. 1999). Recently the expression of LL-37 in NK,  $\gamma\delta$  T cells, B cells and monocytes/macrophages has been described (Agerberth, Charo et al. 2000).

The mature antimicrobial peptides corresponding to the C-terminus are structurally diverse sequences (shown in Fig.1, out of (Popsueva, Zinovjeva et al. 1996)) and individual names have been given to them such as:

bovine cathelicidins (Storici, Tossi et al. 1996), (Skerlavaj, Gennaro et al. 1996), (Gennaro, Scocchi et al. 1998): Bac1 (Bactenecin1), Bac5, Bac7, indolicidin, BMAP-27 (bovine myeloid antimicrobial peptide 27) and BMAP-28;  
porcine cathelicidins (Harwig, Kokryakov et al. 1995): PR-39 (proline-arginine-rich 39-amino-acid peptide), PMAP-36 (porcine myeloid antimicrobial peptide 36), PMAP-37, PMAP-23, protegrins, and prophenins;  
rabbit cathelicidins: CAP18 (cationic antimicrobial protein 18);  
human cathelicidins (Cowland, Johnsen et al. 1995), (Gudmundsson, Agerberth et al. 1996): hCAP-18/FALL-39/LL-37 (human antimicrobial protein/C-terminal derived domains are called FALL-39 or LL-37);  
murine cathelicidins (Gallo, Kim et al. 1997), (Popsueva, Zinovjeva et al. 1996): mCRAMP (murine cathelin-related antimicrobial peptide), MCLP (murine cathelin-like protein);  
rat cathelicidins: rCRAMP (rat cathelin-related antimicrobial peptide);

sheep cathelicidins (Mahoney, Lee et al. 1995), (Bagella, Scocchi et al. 1995): SMAP29 (sheep myeloid antimicrobial peptide 29) and SMAP34.

Beside cathelicidins there are other families of antimicrobial peptides identified in animals and humans, mainly: cecropins and defensins (Gudmundsson and Agerberth 1999), (Boman 2000).

Defensins are a family of 4-kDa peptides and their activity depends on both their net cationic charge as well as their 3-D structure. Defensins form multimeric voltage-dependent pores that permeabilize microbial membranes (Ganz and Lehrer 1994), (Ganz and Lehrer 1999). Although similar in shape to  $\alpha$ -defensins,  $\beta$ -defensins are slightly larger and differ in the placement and connectivity of their six conserved cysteine residues (Ganz and Lehrer 1998).

Human  $\alpha$ -defensins (human neutrophil peptides; HNP.1-4) are mainly found in the granules of neutrophils and participate in the killing of phagocytosed microorganisms (Lehrer, Lichtenstein et al. 1993). More recently two members of this family in humans, HD-5 and HD-6 (human  $\alpha$ -defensins 5 & 6), were found to be constitutively produced by specialized secretory cells in small intestinal crypts, the Paneth cells. HD-5 is also constitutively produced in the female reproductive tract (Ganz and Lehrer 1999).

Two classes of  $\beta$ -defensins can be defined by comparing their expression patterns. Constitutively expressed  $\beta$ -defensins are the human  $\beta$ -defensin 1 (hBD 1), expressed in epithelia, and the bovine neutrophil  $\beta$ -defensin (BNBD-1-13) (Ganz and Lehrer 1998). In contrast, the expression of  $\beta$ -defensins such as bovine lingual antimicrobial peptide (LAP) (Schonwetter, Stolzenberg et al. 1995), the bovine tracheal antimicrobial peptide (TAP) and its human homolog  $\beta$ -defensin 2 (hBD 2) are upregulated during infectious challenge (Ganz and Lehrer 1998). Inducible expression has also been described for other known human beta-defensins hBD-3 and hBD-4 (Harder, Bartels et al. 2000), (Garcia, Krause et al. 2001).

A further class of antimicrobial peptides are cecropins. They were the first antimicrobial peptides found in animals. Bacteria

were shown to induce these compounds in dormant pupae of the giant silk moth *Hyalophora cecropia* (Boman 1991). Their 3D-structure consists of two  $\alpha$ -helices with a hinge in between. Cecropins have so far been found in higher insects and a mammalian cecropin has been isolated from pig intestine (Boman 2000). Cecropin-like peptides have been isolated from sponges and from the *Helicobacter pylori* ribosomal protein L1 (Putsep, Branden et al. 1999), (Putsep, Normark et al. 1999).

Given the very high concentrations that have been recorded at sites of inflammation (Hancock and Diamond 2000) (e.g. 300 $\mu$ g/ml or more in the sputum of cystic fibrosis patients; 20-100 $\mu$ g/ml in the dorsal tongue; up to 170 $\mu$ g/ml in the plasma of septic individuals), one might suspect a key role of CAPs to cope with infections. In addition, CAPs are found at mucosal and epithelial surfaces and in the gut, lungs, kidneys and skin. Their induction during inflammation correlates with a primary role in assisting and/or directing inflammatory responses. Indeed, increased levels of CAPs have been observed in a number of clinical and laboratory-induced infectious and inflammatory states (Hancock and Diamond 2000). Recently, a single enzyme necessary for processing of the pre-prodefensins to the active mature form was identified. Genetic inactivation of this single gene (matrilysin; matrix-metalloproteinase-7: MMP-7) completely inhibited production of active defensin, and subsequently a tenfold increase in the susceptibility to infection by orally introduced virulent bacteria was observed (Wilson, Ouellette et al. 1999). Additionally, a wide range of animal studies and early clinical trials have demonstrated that when exogenously added, naturally and non-naturally occurring CAPs protect against local or systemic infection by bacteria and fungi (reviewed in (Hancock and Diamond 2000), (Hancock 1999)).

However, the action of CAPs is not limited to direct killing of microorganisms. Instead, they have a variety of additional activities that have an impact particularly on the quality and effectiveness of immune responses. CAPs have been reported to be involved in:

- a.) the initial lysis of bacterial cells to release

inflammatory stimuli such as lipopolysaccharides (LPS), lipoteichoic acids (LTA) or CpG (Hancock and Diamond 2000), (Hancock and Scott 2000);

b.) neutralizing LPS and LTA, thus inhibiting the production of TNF- $\alpha$  and IL-6 production by macrophages (= antiseptic activity) (Scott, Rosenberger et al. 2000), (Scott, Yan et al. 1999), (Scott, Gold et al. 1999), (Gough, Hancock et al. 1996);

c.) mast cell degranulation.  
Alpha-defensins have been shown to induce histamine release and vasodilation (Befus, Mowat et al. 1999). In addition, hBD-2 and LL-37 but not hBD-1 were shown to induce histamine release and intracellular calcium mobilization in mast cells. Furthermore, hBD-2 but not LL-37 and hBD-1 exerts prostaglandin D<sub>2</sub> production in mast cells (Niyonsaba, Someya et al. 2001).

d.) inhibition of fibrinolysis by tissue plasminogen activator, thus reducing the spreading of bacteria (Higazi, Ganz et al. 1996);

e.) tissue/wound repair through promotion of fibroblast chemotaxis and growth (Gallo, Ono et al. 1994), (Chan and Gallo 1998);

f.) inhibition of tissue injury by inhibiting certain proteases such as furin and cathepsin (Basak, Ernst et al. 1997), (Van Wetering, Mannesse-Lazeroms et al. 1997);

g.) inhibiting the release of immunosuppressive cortisol (Hancock and Diamond 2000);

h.) the recruitment of various immune cell populations.  
 $\alpha$ -defensins have been shown to induce IL-8 production in airway epithelial cells, leading to a recruitment of neutrophils (Van Wetering, Mannesse-Lazeroms et al. 1997). In addition it has been reported that  $\alpha$ -defensins exert chemotactic activity for naive CD4<sup>+</sup>/CD45RA<sup>+</sup> and CD8<sup>+</sup> T cells, but not for CD4<sup>+</sup>/CD45RO<sup>+</sup> memory T cells. (Chertov, Michiel et al. 1996), (Yang, Chen et

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al. 2000). Similarly,  $\alpha$ -defensins and  $\beta$ -defensins were shown to have the capacity to induce the migration of monocyte-derived immature dendritic cells but not of monocytes and mature dendritic cells (Yang, Chertov et al. 1999), (Yang, Chen et al. 2000). Furthermore, this chemotactic activity of  $\beta$ -defensins was shown to be mediated by interacting with one of the chemokine receptors, CCR6 (chemokine receptor 6) expressed on immature but not on mature dendritic cells (Yang, Chertov et al. 1999). Cathelicidins like the human LL-37 and the porcine PR-39 were shown to exert chemotactic activity for neutrophils (Agerberth, Charo et al. 2000), (De, Chen et al. 2000). Furthermore, LL-37 exert chemotactic activity for CD4<sup>+</sup> T cells but not for CD8<sup>+</sup> T cells (Agerberth, Charo et al. 2000), (De, Chen et al. 2000). In addition it has been shown recently, that LL-37 induces chemotaxis of peripheral blood monocytes, neutrophils and CD4<sup>+</sup> T cells, utilizing the formyl peptide receptor-like 1 (FPRL1) (De, Chen et al. 2000). However, no chemotactic activity of LL-37 for immature and mature dendritic cells was observed. These findings were supported by the fact that with differentiation of monocytes into immature dendritic cells, FPRL1 expression was abolished (Yang, Chen et al. 2001). However, the expression of FPRL1 was described to be restricted to cells of myeloid origin and has not yet been described for T lymphocytes (Murphy 1994). Thus, exerting chemotactic activity for T lymphocytes and cells of myeloid origin like neutrophils and monocytes, LL-37 might utilize different receptors.

i.) the promotion of acquired systemic immune responses. Intranasal delivery of  $\alpha$ -defensins plus ovalbumin (OVA) enhanced OVA-specific serum IgG antibody responses in C57BL/6 mice (Lillard, Boyaka et al. 1999). Furthermore, intraperitoneal administration of keyhole limpet hemocyanin (KLH) adsorbed to the common adjuvant aluminium hydroxide in combination with  $\alpha$ -defensins increased the production of KLH-specific antibodies in Balb/c mice. In addition,  $\alpha$ -defensins enhanced the antibody response to a syngeneic tumor antigen, lymphoma Ig idiotype and also augmented resistance to tumor challenge (Tani, Murphy et al. 2000).

A variety of activities of diverse CAPs (e.g. defensins,

cathelicidins) important for the instruction of adaptive immune responses have been described to date. Common and diverse activities of different CAPs have been figured out. Some clear differences regarding their chemotactic activity for dendritic cells were shown. While  $\alpha$ - &  $\beta$ -defensins chemoattract dendritic cells, chemotactic activity of cathelicidins for this specialized cell type is lacking.

Surprisingly within the scope of the present invention it is shown that cathelicidin derived antimicrobial peptides from different species (the bovine indolicidin, the bovine dodecapeptide, the murine mCRAMP and the human LL-37) exert the ability to enhance the uptake of antigens in dendritic cells of mice and men. Furthermore, subcutaneous administration of a tumor antigen in conjunction with a cathelicidin derived antimicrobial peptide markedly enhanced the immune response to the injected tumor antigen.

In the US 5.837.248 patent, where the stimulation of T cells chemotaxis by a defensin peptide is disclosed, it is mentioned that no other T cell chemotactic peptide would be present in neutrophils apart from defensins and CAP37/azurocidin.

However, even if diverse families of antimicrobial peptides are present in the same cell type (e.g. neutrophils, small intestinal paneth cells; (Ganz and Lehrer 1999)) there are important variations existing among these antimicrobial peptides, which means that features of one family will not necessarily occur in the other family. In general, it appears that the variation is not only due to divergence in amino acid sequences, but also applies to the number and abundance of locally expressed gene products coding for antimicrobial peptides. In the light of this variation, it is clear that these effectors are entities that have been conserved through evolution. Most likely the variation of antibacterial peptides reflects the character of their targets: rapid adaptive evolutionary changes with regard to host-microbe interplay.

The induction of an immune response critically depends on the antigen being available in lymphoid organs. There is no response against antigens that do not reach draining lymph nodes

(Zinkernagel, Ehl et al. 1997). Thus, initiation of immune responses takes place exclusively in lymphoid organs. There, initial interactions between antigen-loaded APCs with T and/or B cells allow the initiation of the immune cascade (Kurts, Heath et al. 1996).

In view of these considerations, immune responsiveness that is increased may simply be a result of enhanced translocation of vaccine antigen from the peripheral site of injection towards the draining local lymph node. In this process, naturally occurring antigen presenting cells residing in the periphery, such as dendritic cells or Langerhans cells, play a central role (Schijns 2000). They are described as "natural adjuvants" because they reside in most tissues as sentinels ready to capture antigen very efficiently, which induces their migration to secondary lymphoid organs where they are capable in priming naive T and B cells (Steinman 1991). They are rapidly recruited into sites of tissue injury in response to inoculation with live or inactivated viruses or bacteria (McWilliam, Napoli et al. 1996).

Despite the fact that cecropins resemble similar cathelicidin-like structural properties ( $\alpha$ -helical conformation), cecropins in contrast to cathelicidins do not show any antigen pulsing capacity (see example). It is surprising that cathelicidin derived antimicrobial peptides have antigen pulsing capacity and therefore immune response stimulating activity. This confirms that different classes of antimicrobial peptides have different functional abilities and that therefore the reported stimulation of T cells chemotaxis by defensin peptides does not indicate for the skilled man in the art that there exists a similar linkage between innate and adaptive immunity.

Thus, cathelicidins and defensins both released at inflamed tissues instruct adaptive immune responses in different ways. While defensins participate by attracting dendritic cells, cathelicidins are the key for the activation of dendritic cells, as shown in the present invention. Therefore, cathelicidins are central components in mediating immune response stimulating activity and therefore constitute highly effective adjuvants for vaccine development.

It has now surprisingly been shown within the course of the

present invention that cathelicidin derived antimicrobial peptides or derivatives thereof have immune response stimulating activity and therefore constitute highly effective adjuvants.

In the scope of the present invention a cathelicidin derived antimicrobial peptide is to be understood as the carboxy-terminal antimicrobial peptide (preferentially but not exclusively encoded by the fourth exon of the cathelicidin gene), followed by the cathelin-like preproregion (preferentially but not exclusively encoded by the first three exons of the cathelicidin gene) of cathelicidins, or derivatives thereof. The Cathelicidin preproregions share high intra-species identity ranging from 75-87% for bovine and 90-97% identity for porcine preproregions (Zanetti, Gennaro et al. 1995), they also possess high inter-species identity ranging from 51-65% (compared to hCAP-18, with the program blastp; (Altschul, Madden et al. 1997)), thus possessing intra- and inter-species homology. In the light of this known high intra- and inter-species protein sequence identity of cathelicidin preproregions, for the present invention all antimicrobial peptides are being termed cathelicidin derived antimicrobial peptide if they are derived from proteins or protein domains which possess a protein-sequence identity to the cathelicidin preproregion of higher than ( $\geq$ ) 45%, advantageously higher than 60%, preferably higher than 80% and still preferred higher than 90%, thus are the antimicrobial domains of these proteins to be understood as cathelicidin derived antimicrobial peptides.

Examples of cathelicidin derived antimicrobial peptides are e.g. PMAP-37, hCAP18, BMAP-27, CAP18, Bac5, Bac7, PR-39, indolicidin, bovine dodecapeptide, protegrin PG-2, etc.

An antimicrobial peptide is being termed antimicrobial or bactericidal when it shows activity in the minimum inhibitory concentration assay (MIC), a routinely used assay (Gudmundsson and Agerberth 1999), (Boman 2000).

The MIC of a substance for a range of microorganisms is preferably determined by the broth dilution method which is a particularly exact method. Serial dilutions of each substance



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are done in Luria-Bertani medium in 96 well plates. Each well is inoculated with 10 $\mu$ l of 10<sup>4</sup> - 10<sup>5</sup> colony-forming-units/ml of the test organism. The MIC is determined after incubation for 36-48 hours of the plates at 37°C. The MIC is taken as the lowest antibiotic concentration at which growth is inhibited.

In the scope of the present invention a cathelicidin derived antimicrobial peptide is being termed antimicrobial or bactericidal if it exerts a MIC below 500  $\mu$ M, preferably below 300  $\mu$ M, still preferred below 200  $\mu$ M, still preferred in a range of between 0.05 and 160  $\mu$ M (Travis, Anderson et al. 2000) of the tested substance to Gram positive and/or Gram negative bacteria, fungi or protozoa.

In the scope of the present invention derivatives of the cathelicidin derived antimicrobial peptides comprise for example fragments of cathelicidin derived antimicrobial peptides as well as cathelicidin derived antimicrobial peptides with one or more mutations such as substitution(s), deletion(s), addition(s), and any modified cathelicidin derived antimicrobial peptides, e.g. salts, esters, etc. Preferably not more than 10% of the amino acids of a given cathelicidin derived antimicrobial peptide according to the present invention shall be substituted, deleted or added. Such mutations are performed according to standard knowledge, e.g. hydrophobic amino acid residues are exchanged by other hydrophobic residues, etc.

A derivative of a cathelicidin derived antimicrobial peptide has to be understood of the cathelicidin molecule as long as the derivative exerts a MIC below 500  $\mu$ M, preferably below 300  $\mu$ M, still preferred below 200  $\mu$ M, still preferred in a range of between 0.05 and 160  $\mu$ M. The length of the cathelicidin derived antimicrobial peptide or derivative thereof according to the present invention is not critical. It may vary from e.g. five amino acids to the length of a protein comprising such an antimicrobial peptide or derivative thereof, preferably between 10 and 60 amino acids, as long as it exerts the above mentioned MIC. The protein is for example a cathelicidin, e.g. MCLP (murine catheline-like protein), hCAP-18, etc. Preferably, the molecules according to the present invention also exhibit

comparable, especially the same or better, chemotactic activities as the naturally occurring cathelicidin derived peptides.

The vaccine comprises at least one cathelicidin derived antimicrobial peptide or a derivative thereof plus at least one antigen the immune response is to be directed against. Of course, the vaccine may comprise two or more antigens depending on the desired immune response. The antigen(s) may also be modified so as to further enhance the immune response.

Preferably, proteins or peptides derived from viral or bacterial pathogens, from fungi or parasites, as well as tumor antigens (cancer vaccines) or antigens with a putative role in autoimmune disease are used as (including derivatized antigens like glycosylated, lipidated, glycolipidated or hydroxylated antigens). Furthermore, carbohydrates, lipids or glycolipids may be used as antigens themselves. The derivatization process may include the purification of a specific protein or peptide from the pathogen, the inactivation of the pathogen as well as the proteolytic or chemical derivatization or stabilization of such a protein or peptide. Alternatively, also the pathogen itself may be used as an antigen. The antigens are preferably peptides or proteins, carbohydrates, lipids, glycolipids or mixtures thereof.

Preferably, the antigen is a peptide consisting of 5 to 60, preferably 6 to 30, especially 8 to 11, amino acid residues. Antigens of this length have been proven to be especially suitable for T cell activation. The antigens can further be coupled with a tail according to A 657/2000. Also, the antigen can be coupled, e.g. covalently bound, to the cathelicidin derived antimicrobial peptide. Of course the resulting compound must not be a naturally occurring cathelicidin.

The relative amounts of the ingredients of the present composition are highly dependent on the necessities of the individual composition. Preferably between 10 ng and 1 g of antigen and cathelicidin derived antimicrobial peptide are applied. Preferred amounts of antigen/cathelicidin derived

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antimicrobial peptide lie in the range of 0.1 to 1000  $\mu\text{g}$  antigen per vaccination and 0.1 to 1000  $\mu\text{g}$  cathelicidin derived antimicrobial peptide.

The composition according to the present invention may further contain auxiliary substances, such as buffers, salts, stabilizers, antioxidants, etc., or other effective substances, such as antiinflammators or antinociceptive drugs.

The present compositions may be applied to a patient, e.g. a vaccination candidate, in efficient amounts, e.g. at weekly, bi-weekly or monthly intervals. Patients to be treated with the present composition may also be vaccinated repeatedly or only once. A preferred use of the present invention is the active immunization, especially of humans or animals without protection against the specific antigen.

The present composition may be applied subcutaneously, intramuscularly, rectally, intravenously, intradermally, intrapinnally, transdermally as well as by oral uptake.

If the vaccine comprises more than one cathelicidin derived antimicrobial peptide or a derivative thereof, these cathelicidin derived antimicrobial peptides will interact with each other so as to enhance the immune response to the antigen(s) even stronger.

Of course, the vaccine according to the present invention can comprise any further substance, as for example any other pharmaceutically acceptable carrier, etc. The vaccine according to the present invention may be formulated according to known methods, e.g. as I.V. vaccines, DNA vaccines, transdermal vaccines, topical vaccines, intranasal vaccines and as combination vaccines. The dosages may be selected by standard processes for vaccines which are improvements of known vaccines, however, a lower dosage than the known vaccine is possible for the same protection and therefore preferred.

Preferably, the vaccine is provided in a storage-stable form, e.g. lyophilized, optionally provided in combination with a

suitable reconstitution solution.

Preferably, the cathelicidin is an animal cathelicidin. In the scope of the present invention "animal cathelicidin" includes human cathelicidin, in particular mammalian cathelicidin. Especially if the cathelicidin is from the animal species for which the vaccine is designed, the antimicrobial peptides derived from these cathelcidins will not be recognized by the animal immune system, thus reducing the risk of mounting an immune response against the antimicrobial peptides derived from cathelcidins in that animal.

According to a preferred embodiment the animal cathelicidin is a mouse cathelicidin, the cathelicidin derived antimicrobial peptide preferably comprising a sequence according to Seq. ID 1. If the vaccine is administered to a mouse, the cathelicidin derived antimicrobial peptide will not be recognized and no immune response against the cathelicidin derived antimicrobial peptide will be induced. However, this cathelicidin derived antimicrobial peptide is also suitable for vaccines which will be administered to any other animal, including humans. The cathelicidin derived antimicrobial peptide comprising the sequence according to Seq. ID 1 has been shown to be particularly effective.

According to a preferred embodiment the cathelicidin is a human cathelicidin, the cathelicidin derived antimicrobial peptide preferably comprising a sequence according to Seq. ID 2. If the vaccine is administered to humans, no immune response against the cathelicidin derived antimicrobial peptide will be induced since it will not be recognized by the immune system. The cathelicidin derived antimicrobial peptide comprising a sequence according to Seq. ID 2 has been shown to be particularly effective when added to a vaccine comprising at least one antigen.

According to a preferred embodiment of the present invention the animal cathelicidin derived antimicrobial peptide is an indolicidin peptide, preferably a bovine indolicidin peptide and particularly preferred comprising a sequence according to Seq.

ID 3.

In the scientific literature different sequences of bovine indolicidin, with (Del Sal, Storici et al. 1992), (Zanetti, Gennaro et al. 1995), (Zanetti, Gennaro et al. 1997) and without (Selsted, Novotny et al. 1992), (Falla, Karunaratne et al. 1996), (Andreu and Rivas 1998), (Hancock and Diamond 2000) a carboxy-terminal glycine, have been published. The tryptophan-rich bovine indolicidin has been purified from bovine neutrophils as an amidated tridecapeptide (Selsted, Novotny et al. 1992). An additional glycine, not found in purified indolicidin, was found present at the carboxyl terminus of the deduced cDNA sequence, likely being involved in post-translational amidation (Del Sal, Storici et al. 1992). In the present invention, bovine indolicidin is preferably synthesized according to the peptide purified from bovine neutrophils (Selsted, Novotny et al. 1992) comprising the sequence according to SEQ ID No. 3 in its C-terminal aminated form: NH<sub>2</sub>-ILPWKWPWWPWR-CONH<sub>2</sub>. This cathelicidin derived antimicrobial peptide is particularly for vaccines designed for bovines, since no immune response will be induced against the cathelicidin derived antimicrobial peptide in this animal species. However, it is also suitable for vaccines for any other animal species, including humans. The cathelicidin derived antimicrobial peptide comprising a sequence according to Seq. ID 3 has proved to be particularly effective as an adjuvant.

A preferred animal cathelicidin derived antimicrobial peptide is a bovine cyclized and/or linear dodecapeptide comprising a sequence according to Seq. ID 4. Even though this cathelicidin derived antimicrobial peptide is rather short, it has been shown to effectively enhance the immune response against the antigen(s) comprised in the vaccine.

Theoretically a vaccine should contain at least two components: (1) the antigen against which the immune response should be mounted and (2) the adjuvant, which is there to enhance and/or direct the immune response. Immunological adjuvants were originally described as "substances used in combination with a specific antigen that produce more immunity than the antigen

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alone" (Singh and O'Hagan 1999). It is known that a high diversity of adjuvants regarding their immunostimulatory capacity is given (Schijns 2000). Thus, improved efficiency has been described in combining differentially acting adjuvants for the preparation of vaccines. For example, protective immunity using the recombinant human cytokine IL-12 and aluminium hydroxide in a primate model of cutaneous leishmaniasis was shown (Kenney, Sacks et al. 1999). Furthermore, improved efficiency of dendritic cell vaccines and successful immunization with tumor antigen peptide-pulsed peripheral blood mononuclear cells by coadministration of the recombinant murine cytokine IL-12 was shown (Fallarino, Uyttenhove et al. 1999). But not only cytokines in combination with other adjuvants were shown to synergize. For example dimethyl dioctadecyl ammoniumbromide coadjuvanted with poly(I-C) or the cytokines IFN- $\gamma$ , IL-2 and IL-12 shows modulating effect of immune responses to tuberculosis subunit vaccines (Lindblad, Elhay et al. 1997).

Preferably, the vaccine comprises at least one further immune response stimulating substance. As immune response stimulating substance any substance or molecule can be used which is known to be active as an adjuvant. Such substances are disclosed in WO93/19768. Other substances may be e.g. polycations, as for example polylysine or polyarginine. Other adjuvants may be components in the form of particles, e.g. silicagel or dextran beads, which are sufficiently small so that they can enter into the cells. The addition of this further immune response stimulating substance will render the vaccine even more efficient.

Preferably, the immune response stimulating substance is a cytokine. Cytokines play an important role in activating and stimulating B cells, T cells and NK cells, macrophages, dendritic cells and various other cells participating in inducing immune responses. Any cytokine can be used which will additionally enhance the immune response to the antigen(s).

Another aspect of the present invention is the use of a cathelicidin derived antimicrobial peptide or a derivative thereof for the preparation of an adjuvant for enhancing the

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immune response to at least one antigen. Also according to this aspect of the invention "cathelicidin derived antimicrobial peptide", "cathelicidin", "derivative", and "antigen" are to be understood as above defined.

Preferably, the adjuvant enhances the uptake of at least one antigen in antigen presenting cells (APC). Since more antigen is taken up in the antigen presenting cells, the APC-induced cascades leading to the induction of antigen specific immune effector cells, like T cells, are enhanced. Therefore, an enhanced uptake of the antigen in APCs enhances the immune response to these antigens.

Preferably, the cathelicidin is an animal cathelicidin. Particularly preferred are cathelicidins which do not induce an immune response in the individual to which the cathelicidins are administered.

According to a preferred embodiment of the present invention the cathelicidin is a mouse cathelicidin, the cathelicidin derived antimicrobial peptide preferably comprising a sequence according to Seq. ID 1.

According to a further advantageous embodiment the cathelicidin is a human cathelicidin, the cathelicidin derived antimicrobial peptide preferably comprising a sequence according to Seq. ID 2.

Preferably, the cathelicidin derived antimicrobial peptide is an indolicidin peptide, preferably a bovine indolicidin peptide and further preferred a cathelicidin derived antimicrobial peptide comprising a sequence according to Seq. ID 3.

Preferably, the cathelicidin derived antimicrobial peptide is a bovine cyclized and/or linear dodecapeptide comprising a sequence according to Seq. ID 4.

The advantages of these above mentioned cathelicidin derived antimicrobial peptide are the same as mentioned above.

According to a preferred embodiment of the invention, the

adjuvant is added to a vaccine. It is of course possible to administer the adjuvant directly to the animal, e.g. preferably before the vaccination. It is, however, easier for the administration to add the adjuvant to a vaccine which is then administered to the animal all at once.

According to a further aspect, the present invention relates to a method of vaccinating an animal including humans against a specific antigen or a group of specific antigens, said method comprising the administration of an effective amount of a vaccine according to the present invention to said animal, including humans, to be vaccinated. Alternatively, the method comprises administering an effective amount of an adjuvant comprising a cathelicidin derived antimicrobial peptide, after which a vaccine is administered.

The invention will be described in more detail by the following examples and figures, but the invention is of course not limited thereto.

Fig.1 shows sequence similarities between cathelicidin proteins.

Fig.2 shows the arrangement for the human gene for proFALL-39/hCAP18.

Fig.3 shows a schematic representation of propeptides of the cathelicidin family.

Fig.4 shows TRANSloading of P388D1 with cathelicidin derived antimicrobial peptides as "carrier-peptides".

Fig.5 shows TRANSloading of P388D1 with cecropin or cathelicidin derived antimicrobial peptides as "carrier-peptides".

Fig.6 shows TRANSloading of P388D1 with increasing amounts of cathelicidin derived antimicrobial peptides (bovine linear dodecapeptide SEQ ID 4) as "carrier-peptide".

Fig.7 shows TRANSloading of P388D1 with increasing amounts of cecropin like Hp RpL1 (amino acid residues 2-20) derived



antimicrobial peptides as "carrier-peptides".

Fig.8 shows TRANSloading of human DCs with human MHC class I and MHC class II peptides by LL-37.

Figures 9 and 10 show the amount of IFN- $\gamma$ -producing cells in vaccinated mice.

In Fig.1 the similarities of various cathelicidin proteins are shown. The deduced amino acid sequence of MCLP (murine catheline-like protein, SEQ. ID 5) is aligned with the precursor sequence of peptide antibiotics from rabbit (cathelin, SEQ. ID 6, and CAP18, SEQ. ID 7), cow (bactenecin, SEQ. ID 8, Bac5, SEQ. ID 9, indolicidin, SEQ. ID 10), human (FALL-39, SEQ. ID 11). These are examples of cathelicidin derived antimicrobial peptides according to the present invention. Cysteins are boxed. The dibasic protease processing sites are underlined. Alignment was performed using the program DNA-SUN.

Fig.2 shows the arrangement of the human gene for proFALL-39/hCAP18. The overall structure with three conserved exons (e1-e3) is the same for all cathelicidin genes. The variable part is always exon 4, which in human, pig, cow, rabbit, mouse and sheep can code for totally different effectors, belonging to the first four classes of antimicrobial peptides. Region 1 indicates the control sites for transcription factors like NF-KB, NF-IL6, APRF. Arrow 2 indicates the hypothetic site for exon shuffling, region 3 shows the signal peptide, region 4 the cathelin-derived precursor, region 5 the primary translation product. Region 6 indicates the product of exon 4 wherein FALL-39 and LL-37 are the abbreviations for the C-terminal antimicrobial peptides derived from hCAP-18. The processing of FALL-39 has not yet been worked out.

Fig.3 shows the schematic representation of propeptides of the cathelicidin family. Some of the C-terminal antimicrobial peptides are shown, representative of  $\alpha$ -helical (PMAP-37, SEQ. ID 12; hCAP18, SEQ. ID 13; BMAP-27, SEQ. ID 14; CAP18, SEQ. ID 15), Pro- and Arg-rich (Bac5, SEQ. ID 16; Bac7, SEQ. ID 17; PR-39, SEQ. ID 18), Trp-rich (indolicidin, SEQ. ID 3), one

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disulfide bridge containing(dodecapeptide, SEQ. ID 4), and two disulfide bridges containing (protegrin PG-2, SEQ. ID 19) sequences. These are examples for cathelicidin derived antimicrobial peptides according to the present invention. Region 1 indicates the conserved pre-proregion, region 2 the variable antimicrobial domain, arrow 3 indicates the site for the signal peptidase, arrow 4 the cleaving site for elastase. The numbers under the three regions (pre, pro, pep(tide)) indicate the number of amino acid residues of the peptides.

#### **E X A M P L E S**

**Test for the ability of different peptides to enhance the uptake of a labelled antigenic peptide into APCs (TRANSloading assay; (Buschle, Schmidt et al. 1997)) and the induction of peptide specific T cell responses in vivo**

To test if diverse cathelicidin or cecropin derived antimicrobial peptides are able to function as "carrier-peptides" for antigens, to TRANSload APCs in vitro, which means enhancing the antigen uptake into APCs, fluorescent labelled peptides were used as antigenic peptides. They were mixed with diverse types and concentrations of "carrier-peptides" as indicated.

To compare the efficiency of peptide delivery of these diverse "carrier-peptides", the amount of peptide uptake into APCs was monitored by incubating P388D1 cells (murine monocyte-macrophage antigen presenting cell line; purchased from ATCC (TIB-63), or human CD1a positive (derived from human HLA-A2 positive donors, CD14+ positive PBMCs) dendritic cells, for 1h at 37°C with a constant amount of fluorescein-tagged peptide alone or in combination with diverse "carrier-peptides" at concentrations indicated. Before analysing the cells by flow cytometry, the cells were washed extensively to remove free peptide. The relative amount of fluorescein-tagged peptide taken up by the cells was measured by flow-cytometry.

**Example 1****TRANSloading murine macrophages with cathelicidin derived antimicrobial peptides as "carrier-peptides"**

Bovine indolicidin (SEQ ID 3), linear or cyclized bovine dodecapeptide (SEQ ID 4), murine cathelicidin derived antimicrobial peptide (SEQ ID 1) were used at concentrations representing an equal amount of positive charges. The antigenic peptide used is an influenza-haemagglutinin derived MHC class I (Kd) binding peptide (Buschle, Schmidt et al. 1997). The amounts of antigenic peptide and carrier-peptides used were as follows (see Fig.4, fluorescence intensity in log scale):

- (1) No peptide (cells alone)
- (2) 2µg FL-LFEAIEGFI (peptide alone)
- (3) 2µg FL-LFEAIEGFI + 63µg bovine indolicidin (SEQ ID 3)
- (4) 2µg FL-LFEAIEGFI + 75µg cyclized bovine dodecapeptide (SEQ ID 4)
- (5) 2µg FL-LFEAIEGFI + 75µg linear bovine dodecapeptide (SEQ ID 4\*)
- (6) 2µg FL-LFEAIEGFI + 20µg poly-L-arginine
- (7) 2µg FL-LFEAIEGFI + 58µg murine antimicrobial peptide (SEQ ID 1)

Whereas fluorescence appears to be sparse in cells treated with peptide alone, intense fluorescence of "TRANSloaded" cells was found in all cells which were TRANSloaded with cathelicidin derived antimicrobial peptides as "carrier-peptides", indicating that they are able to pulse APCs with antigenic peptides very efficiently. All tested cathelicidin derived antimicrobial peptides greatly enhance the peptide delivery and function as good "carrier-peptide" to APCs.

**Example 2****A comparison of cecropins and cathelicidin derived antimicrobial peptides for their TRANSloading activity**

Bovine indolicidin (SEQ ID 3), linear or cyclized bovine

dodecapeptide (SEQ ID 4) and cecropin like *Helicobacter pylori* RpL1 derived peptide (Hp RpL1 2-20); amino acid residues 2-20 (Putsep, Normark et al. 1999), (Boman 2000), were used at concentrations representing an equal amount of positive charges. The antigenic peptide used is an influenza-haemagglutinin derived MHC class I (Kd) binding peptide (Buschle, Schmidt et al. 1997). The amounts of antigenic peptide and carrier-peptides used were as follows:

- (1) No peptide (cells alone)
- (2) 2 $\mu$ g FL-LFEAIEGFI (peptide alone)
- (3) 2 $\mu$ g FL-LFEAIEGFI + 47 $\mu$ g cecropin like Hp RpL1 2-20
- (4) 2 $\mu$ g FL-LFEAIEGFI + 63 $\mu$ g bovine indolicidin (SEQ ID 3)
- (5) 2 $\mu$ g FL-LFEAIEGFI + 37,5 $\mu$ g cyclized bovine dodecapeptide (SEQ ID 4)

While cathelicidin derived antimicrobial peptides show clear and significant TRANSloading activity, cecropin derived antimicrobial peptides exert only little enhancement of peptide uptake (s. Fig. 5, fluorescence intensity in log scale).

### Example 3

#### Linear bovine dodecapeptide at increasing concentrations

The antigenic peptide used is an influenza-haemagglutinin derived MHC class I (Kd) binding peptide (Buschle, Schmidt et al. 1997). The amounts of antigenic peptide and carrier-peptides used were as follows.

- (1) No peptide (cells alone)
- (2) 2 $\mu$ g FL-LFEAIEGFI (peptide alone)
- (3) 2 $\mu$ g FL-LFEAIEGFI + 18.75 $\mu$ g lin. bovine dodecapeptide (SEQ ID 4)
- (4) 2 $\mu$ g FL-LFEAIEGFI + 37,5 $\mu$ g lin. bovine dodecapeptide (SEQ ID 4)
- (5) 2 $\mu$ g FL-LFEAIEGFI + 75 $\mu$ g lin. bovine dodecapeptide (SEQ ID 4)
- (6) 2 $\mu$ g FL-LFEAIEGFI + 150 $\mu$ g lin bovine dodecapeptide (SEQ ID 4)

It was shown (Figure 6, fluorescence intensity in log scale)

that with increasing amounts of cathelicidin derived antimicrobial peptides (bovine dodecapeptide: SEQ ID 4) the pulsing effect also increased significantly.

#### Example 4

##### **Cecropin like Hp RpL1 derived antimicrobial peptide at increasing concentrations**

The antigenic peptide used is an influenza-haemagglutinin derived MHC class I (Kd) binding peptide (Buschle, Schmidt et al. 1997). The amounts of antigenic peptide and carrier-peptides used were as follows (see Fig.7, fluorescence intensity in log scale).

- (1) No peptide (cells alone)
- (2) 2µg FL-LFEAIEGFI (peptide alone)
- (3) 2µg FL-LFEAIEGFI + 25µg cecropin like Hp RpL1 2-20
- (4) 2µg FL-LFEAIEGFI + 50µg cecropin like Hp RpL1 2-20
- (5) 2µg FL-LFEAIEGFI + 100µg cecropin like Hp RpL1 2-20
- (6) 2µg FL-LFEAIEGFI + 200µg cecropin like Hp RpL1 2-20

Fig.7 shows that increasing amounts of cecropin derived antimicrobial peptides do not effectively increase the pulsing effect.

#### Example 5

##### **TRANSloading of human dendritic cells with a MHC class I and MHC class II peptides by LL-37**

To show that not only murine APCs but also human APCs are TRANSloaded by cathelicidin derived antimicrobial peptides, human CD1a positive (derived from human HLA-A2 positive donors, CD14+ positive PBMCs) dendritic cells were used as target APCs, and they were pulsed with a MHC class I binding peptide derived from influenza matrix protein A (amino acid residues 58-67, (Morrison, Elvin et al. 1992) or a MHC class II binding peptide derived from tetanus toxin (amino acid residues 830-843, (Valmori, Sabbatini et al. 1994). These two classes of antigenic fluorescein-tagged peptides were used. As cathelicidin derived

antimicrobial peptide from human the known LL-37 (SEQ ID 2) peptide (Cowland, Johnsen et al. 1995) was used.

The concentration of human cathelicidin derived antimicrobial peptide LL-37 and the antigenic peptides used are indicated as follows:

Fig. 8a

- (1) no peptide (cells alone)
- (2) 2,5  $\mu$ g FL-GILGFVFTLT (MHC class I; peptide alone)
- (3) 2,5 $\mu$ g FL-GILGFVFTLT (MHC class I) + 30 $\mu$ g LL-37 (SEQ ID 2)

Fig. 8b

- (1) no peptide (cells alone)
- (2) 2,5  $\mu$ g FL-QYIKANSKFIGITE (MHC class II; peptide alone)
- (3) 2,5 $\mu$ g FL-QYIKANSKFIGITE (MHC class II) + 30 $\mu$ g LL-37 (SEQ ID 2)

As shown in Fig. 8a and 8b, the human cathelicidin derived antimicrobial peptide, LL-37 pulsed human dendritic cells with both classes (MHC class I & MHC class II) of antigenic peptide to a significant extent.

Thus, cathelicidin derived antimicrobial peptides from diverse species can serve as "carrier peptides" to pulse APCs of different origins.

#### Example 6

#### Testing the ability to enhance the induction of peptide specific T cells responses in vivo

For testing the ability of these cathelicidin derived antimicrobial peptides to enhance the induction of peptide specific T cell responses in vivo, groups of 4 mice (C57BL/6, female, 8 weeks of age, H-2b) were injected subcutaneously into the flank 3 times (days 0, 7, and 14), by using a mixture of an antigenic melanoma peptide (100 $\mu$ g) derived from TRP-2 (mouse tyrosinase related protein-2: amino acid sequence: 181-188; VYDFFVWL) (Bloom, Perry-Lalley et al. 1997) and diverse

"carrier-peptides", either poly-L-arginine, murine cathelicidin derived antimicrobial peptide (SEQ ID 1) or bovine indolicidin (SEQ ID 3). The groups of mice were injected as follows (amounts indicated/per mouse).

- (1) 100 $\mu$ g VYDFFVWL
- (2) 100 $\mu$ g VYDFFVWL + 100 $\mu$ g poly-L-arginine
- (3) 100 $\mu$ g VYDFFVWL + 1000 $\mu$ g murine cathelicidin derived antimicrobial peptide (SEQ ID 1)
- (4) 100 $\mu$ g VYDFFVWL + 500 $\mu$ g bovine indolicidin (SEQ ID 3)

Two weeks after the 3<sup>rd</sup> vaccination, draining (inguinal) lymph nodes and spleens were removed and lymph node cells (Figure 9) and splenocytes (Figure 10) were activated ex vivo with TRP-2 derived (mouse tyrosinase related protein-2: aminoacid sequence 181-188: VYDFFVWL) peptide to determine IFN- $\gamma$ -producing specific cells in an ELISpot assay (number of IFN- $\gamma$  ELISpots per million splenocytes and lymph node cells, respectively).

Fig. 9 shows that an injection of mice with peptide plus bovine indolicidin (SEQ ID 3) resulted in more IFN- $\gamma$ -producing specific cells than an injection of mice only with peptide or with peptide plus poly-L-arginine.

Fig. 10 shows that both groups of mice injected with peptide plus bovine indolicidin (SEQ ID 3) and murine cathelicidin derived antimicrobial peptide (SEQ ID 1) had more IFN- $\gamma$ -producing specific cells than mice injected only with peptide or with peptide plus poly-L-arginine.

This examples demonstrate clearly that cathelicidin derived antimicrobial peptides enhance the induction of peptide specific T cell responses in vivo.

In summary, all of the tested cathelicidin derived antimicrobial peptides showed a high "TRANSloading" and immunostimulating efficiency, indicating that cathelicidin derived antimicrobial peptides are able to pulse APCs with antigenic peptides in vitro and in vivo very efficiently and are good adjuvants/"carrier-peptides" for antigenic peptides in inducing adaptive immune

responses.



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## C l a i m s :

1. Vaccine, characterized in that it comprises at least one antigen and at least one cathelicidin derived antimicrobial peptide or a derivative thereof.
2. Vaccine according to claim 1, characterized in that the cathelicidin is an animal cathelicidin.
3. Vaccine according to claim 2, characterized in that the cathelicidin is a mouse cathelicidin.
4. Vaccine according to claim 3, characterized in that the mouse cathelicidin derived antimicrobial peptide comprises a sequence according to Seq. ID 1.
5. Vaccine according to claim 2, characterized in that the cathelicidin is a human cathelicidin.
6. Vaccine according to claim 5, characterized in that the human cathelicidin derived antimicrobial peptide comprises a sequence according to Seq. ID 2.
7. Vaccine according to claim 2, characterized in that the animal cathelicidin derived antimicrobial peptide is an indolicidin peptide.
8. Vaccine according to claim 7, characterized in that the indolicidin peptide is a bovine indolicidin peptide.
9. Vaccine according to claim 8, characterized in that the bovine indolicidin peptide comprises a sequence according to Seq. ID 3.
10. Vaccine according to claim 2, characterized in that the animal cathelicidin derived antimicrobial peptide is a bovine cyclized and/or linear dodecapeptide comprising a sequence according to Seq. ID 4.
11. Vaccine according to any one of claims 1 to 10,

characterized in that it comprises at least one further immune response stimulating substance.

12. Vaccine according to claim 11, characterized in that the immune response stimulating substance is a cytokine.

13. The use of a cathelicidin derived antimicrobial peptide or a derivative thereof for the preparation of an adjuvant for enhancing the immune response to at least one antigen.

14. The use according to claim 13, characterized in that the adjuvant enhances the uptake of at least one antigen in antigen presenting cells (APC).

15. The use according to claim 13 or 14, characterized in that the cathelicidin is an animal cathelicidin.

16. The use according to claim 15, characterized in that the cathelicidin is a mouse cathelicidin.

17. The use according to claim 16, characterized in that the cathelicidin derived antimicrobial peptide comprises a sequence according to Seq. ID 1.

18. The use according to claim 15, characterized in that the cathelicidin is a human cathelicidin.

19. The use according to claim 18, characterized in that the cathelicidin derived antimicrobial peptide comprises a sequence according to Seq. ID 2.

20. The use according to claim 15, characterized in that the cathelicidin derived antimicrobial peptide is an indolicidin peptide.

21. The use according to claim 20, characterized in that the cathelicidin derived antimicrobial peptide is a bovine indolicidin peptide.

22. The use according to claim 21, characterized in that the

cathelicidin derived antimicrobial peptide comprises a sequence according to Seq. ID 3.

23. The use according to claim 15, characterized in that the cathelicidin derived antimicrobial peptide is a bovine cyclized and/or linear dodecapeptide comprising a sequence according to Seq. ID 4.

24. The use according to one of claims 13 to 23, characterized in that the adjuvant is added to a vaccine.



2/10

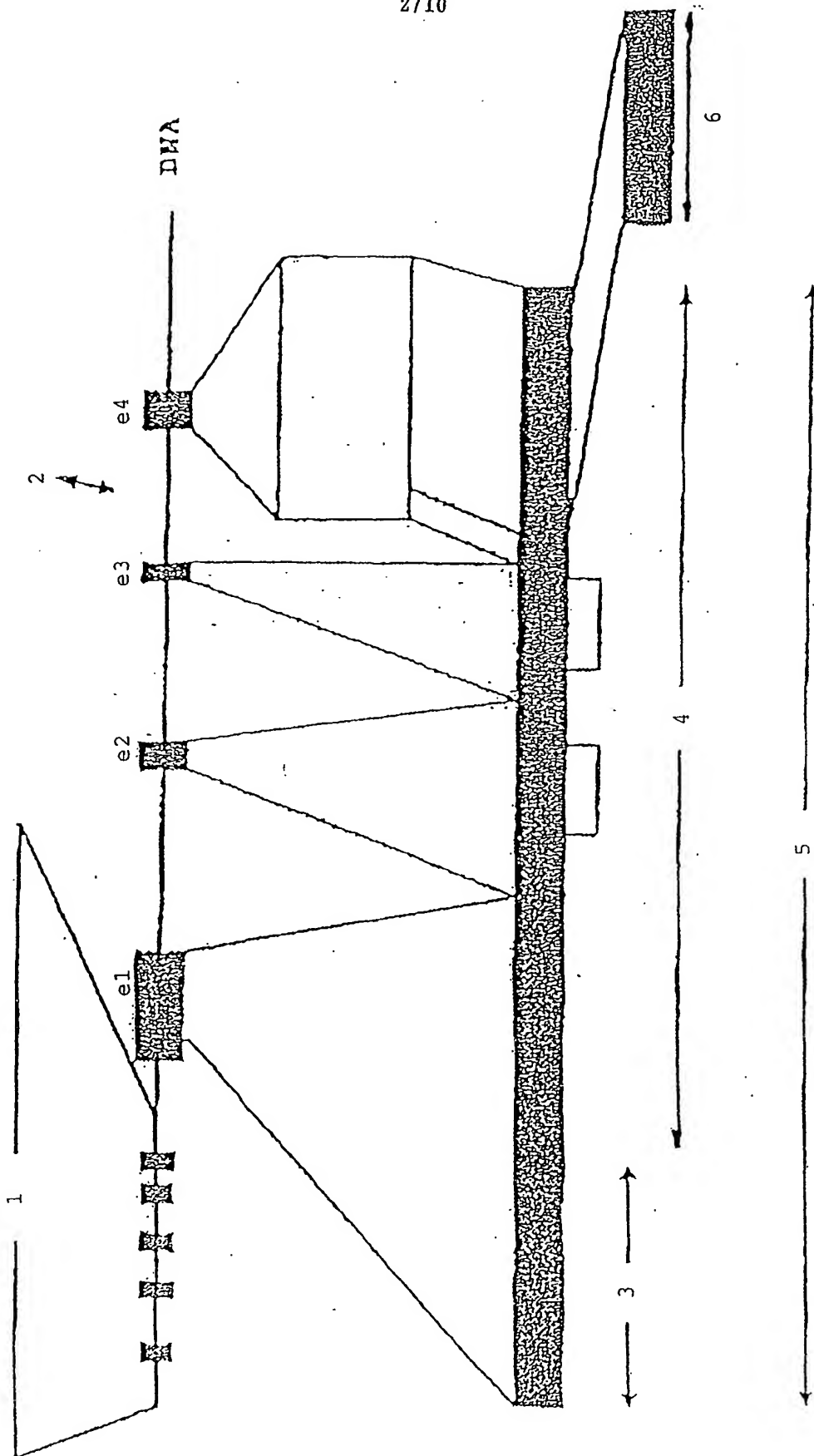


Fig. 2

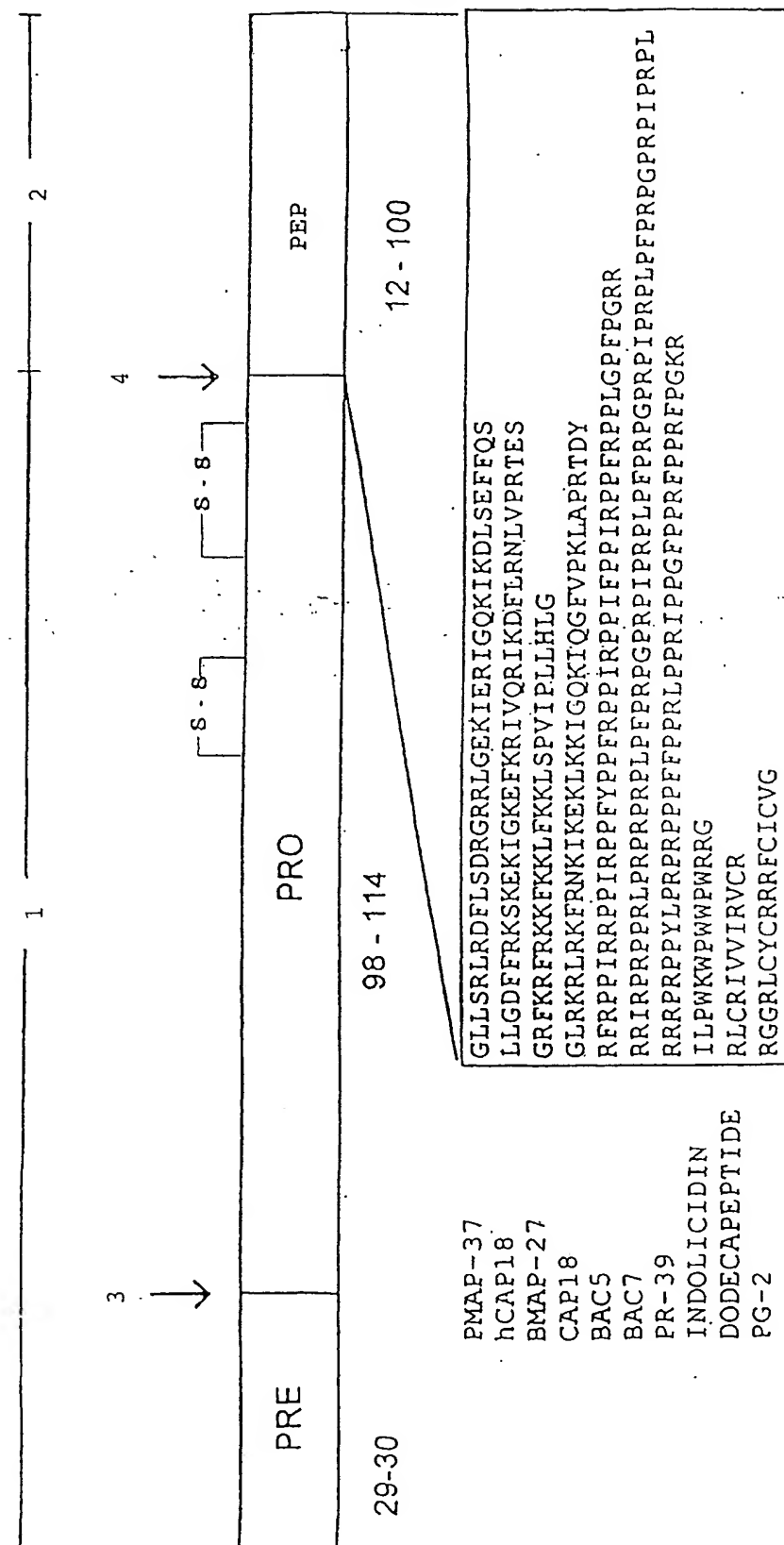


Fig. 3



Fig. 4

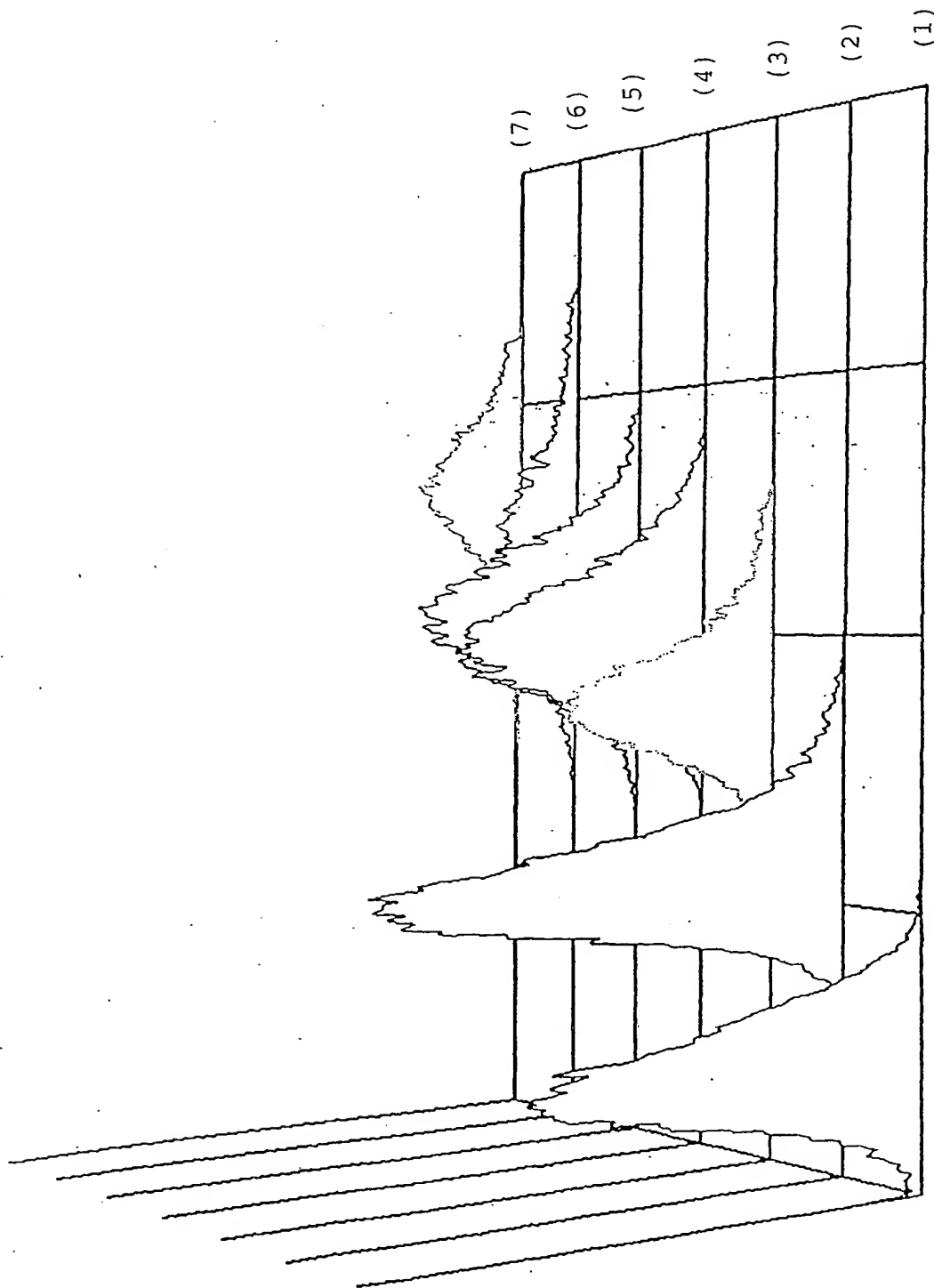


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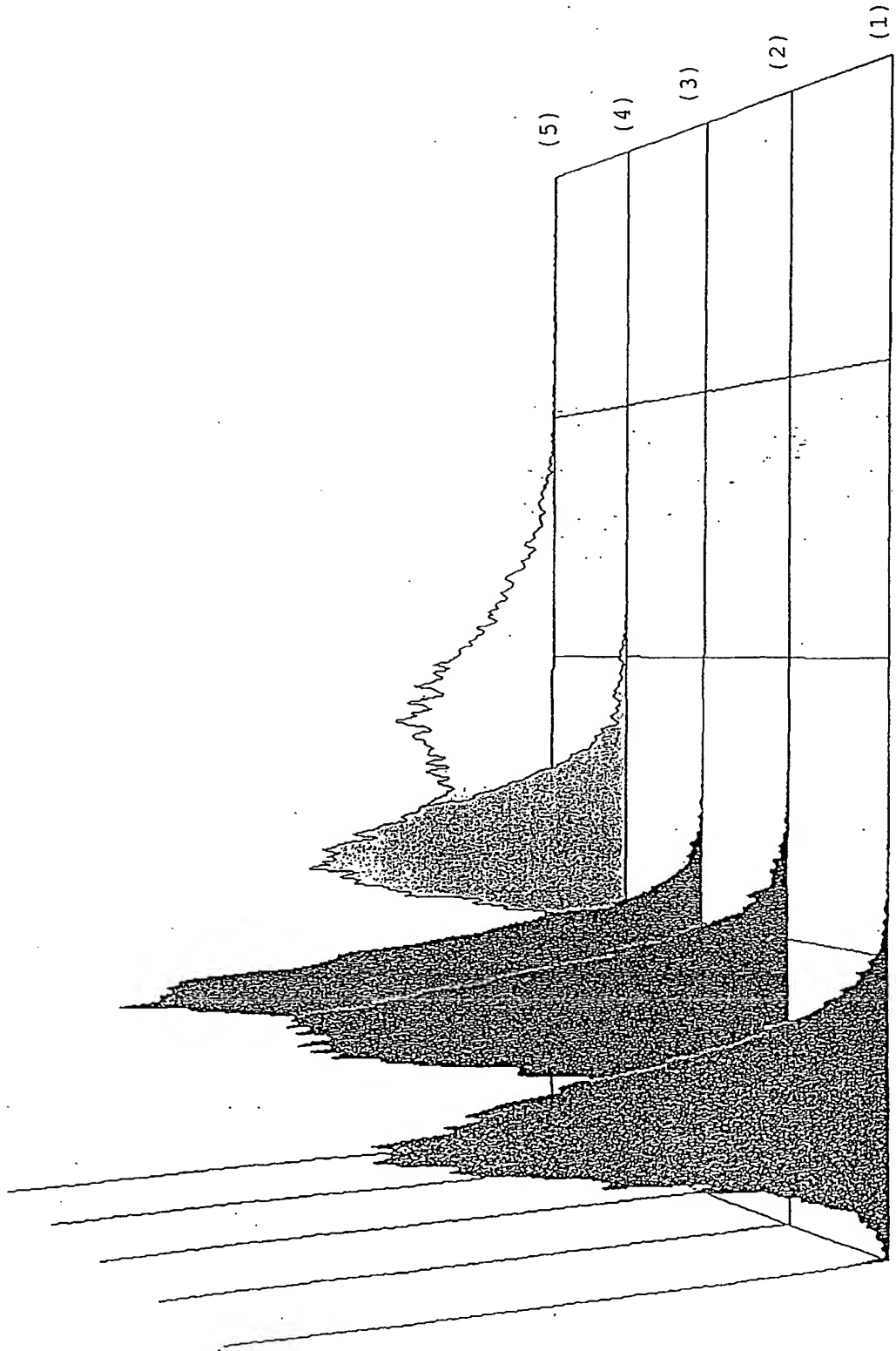


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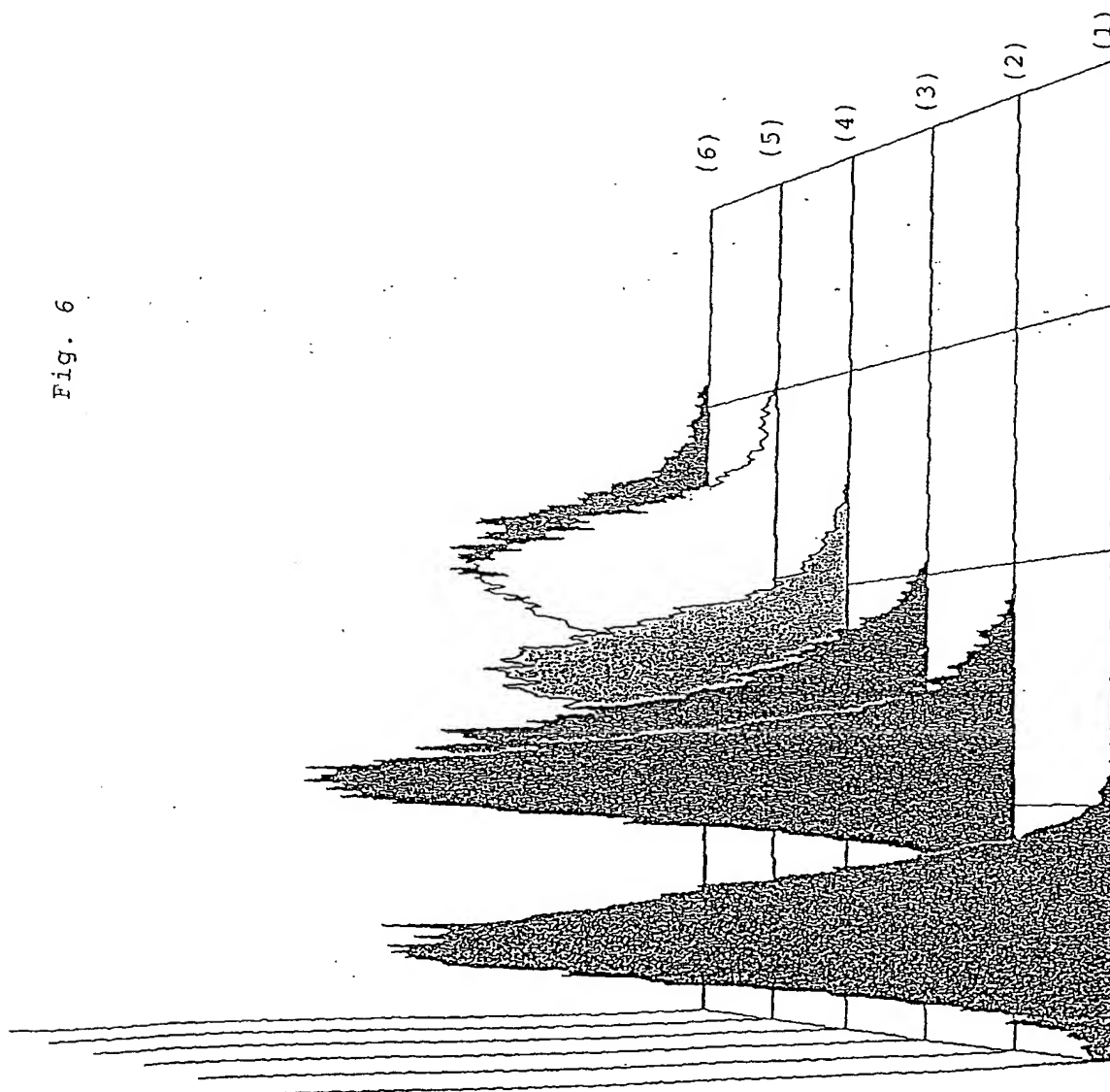


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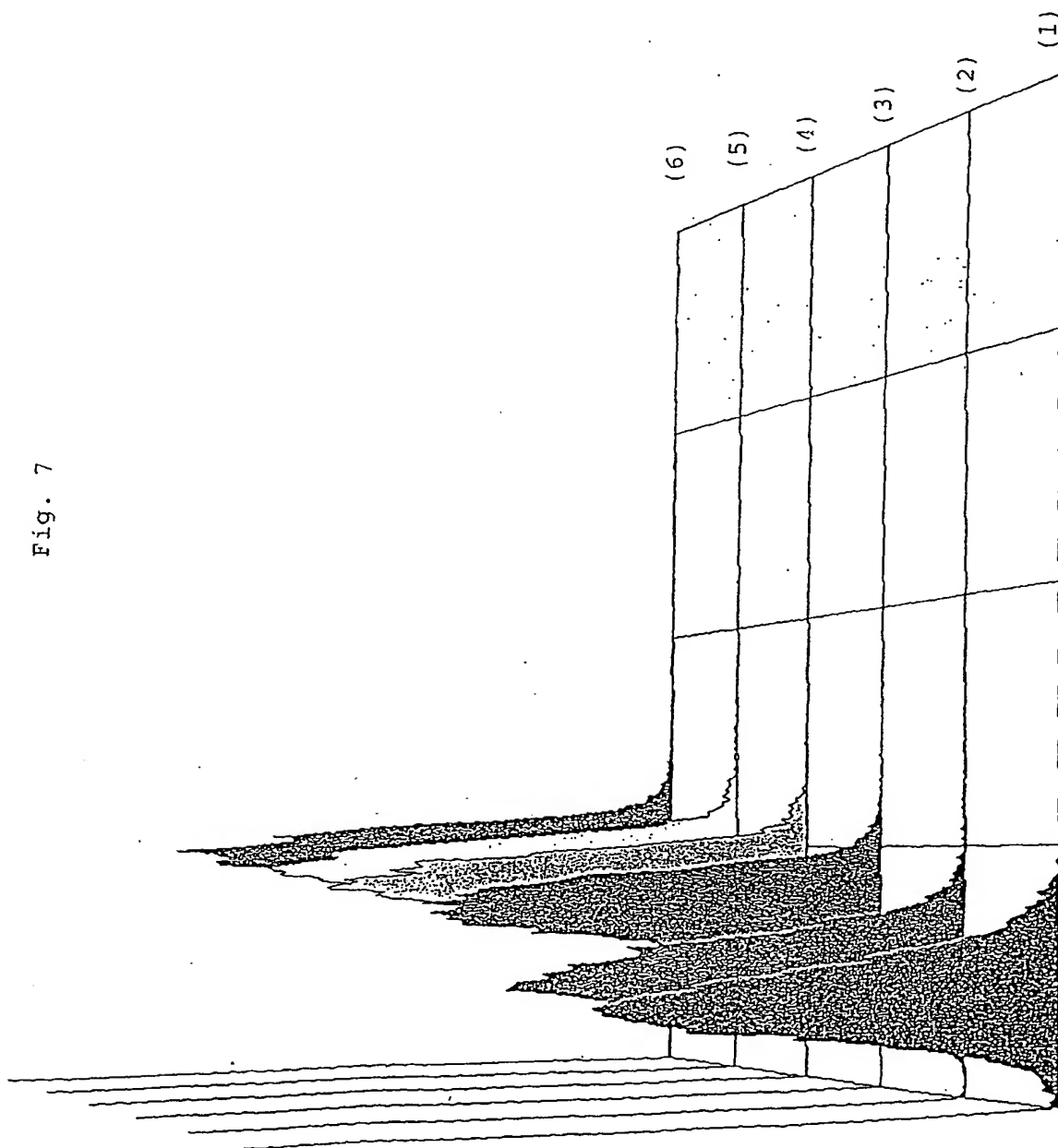


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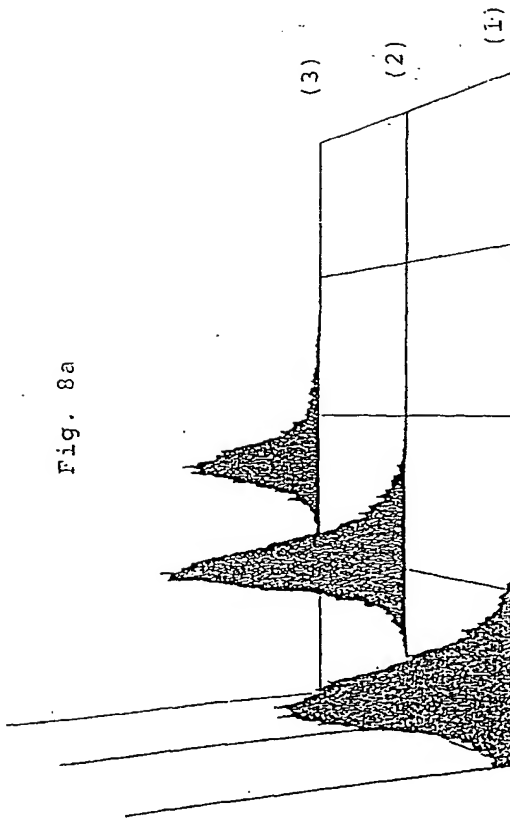


Fig. 8b

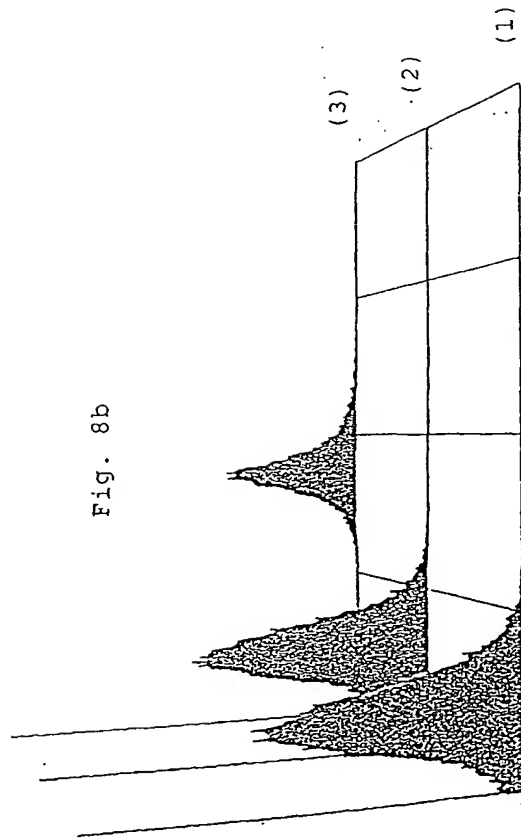


Fig. 9

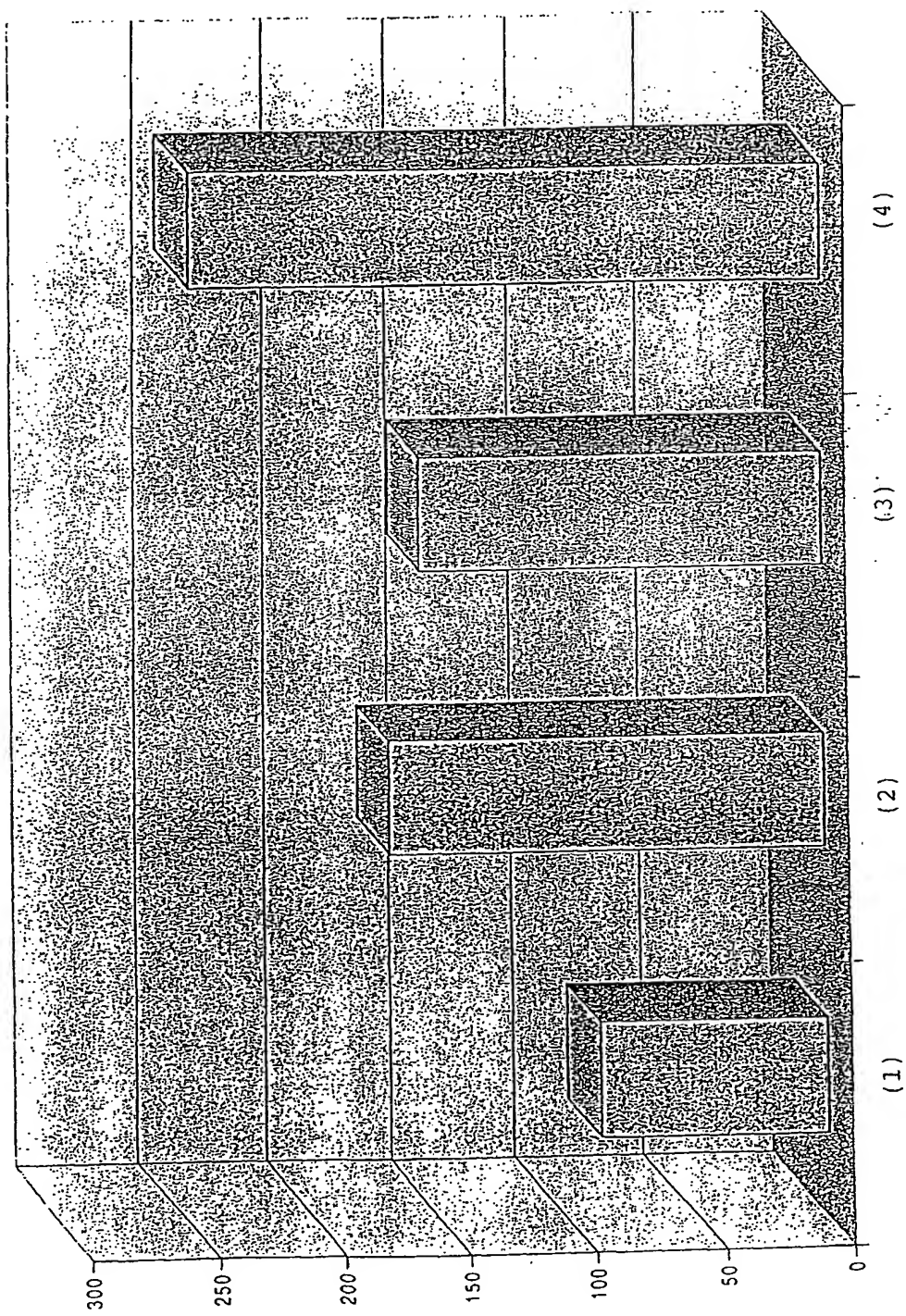
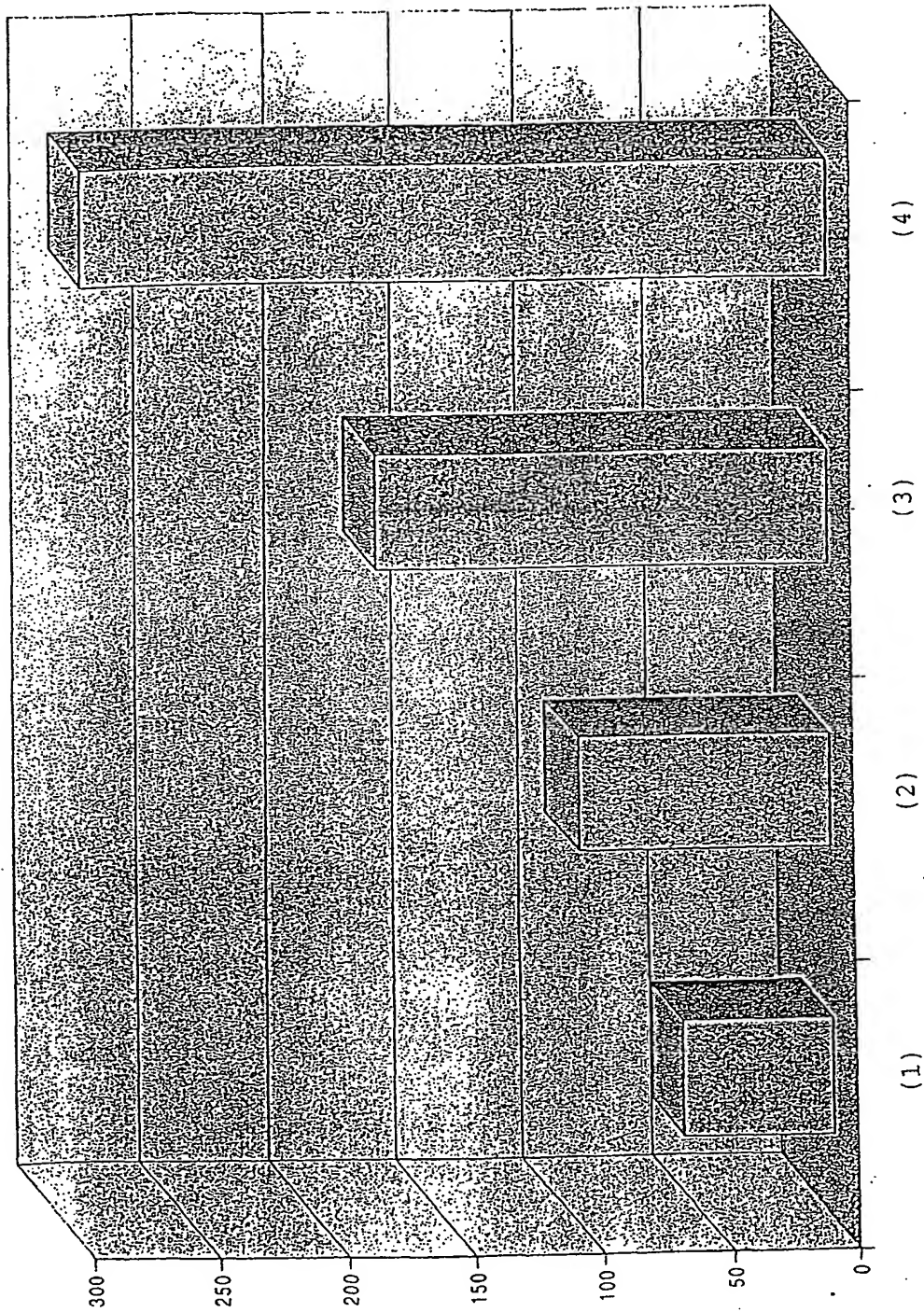


Fig. 10



## SEQUENZPROTOKOLL

<110> Cistem Biotechnologies GmbH

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Gly Leu Leu Ser Arg Leu Arg Asp Phe Leu Ser Asp Arg Gly Arg Arg  
 1 5 10 15

Leu Gly Glu Lys Ile Glu Arg Ile Gly Gln Lys Ile Lys Asp Leu Ser  
 20 25 30

Glu Phe Phe Gln Ser  
 35

&lt;210&gt; 13

&lt;211&gt; 37

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:peptide

&lt;400&gt; 13

Leu Leu Gly Asp Phe Phe Arg Lys Ser Lys Glu Lys Ile Gly Lys Glu  
 1 5 10 15

Phe Lys Arg Ile Val Gln Arg Ile Lys Asp Phe Leu Arg Asn Leu Val  
 20 25 30

Pro Arg Thr Glu Ser  
 35

&lt;210&gt; 14

&lt;211&gt; 27

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:peptide

&lt;400&gt; 14

Gly Arg Phe Lys Arg Phe Arg Lys Lys Phe Lys Lys Leu Phe Lys Lys  
 1 5 10 15

Leu Ser Pro Val Ile Pro Leu Leu His Leu Gly  
 20 25

&lt;210&gt; 15

&lt;211&gt; 37

&lt;212&gt; PRT

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:peptide

&lt;400&gt; 15

Gly Leu Arg Lys Arg Leu Arg Lys Phe Arg Asn Lys Ile Lys Glu Lys  
 1 5 10 15

Leu Lys Lys Ile Gly Gln Lys Ile Gln Gly Phe Val Pro Lys Leu Ala  
 20 25 30

Pro Arg Thr Asp Tyr

35

<210> 16  
 <211> 46  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:peptide

<400> 16  
 Arg Phe Arg Pro Pro Ile Arg Arg Pro Pro Ile Arg Pro Pro Phe Tyr  
           1                          5                          10                          15  
 Pro Pro Phe Arg Pro Pro Ile Arg Pro Pro Ile Phe Pro Pro Ile Arg  
                           20                          25                          30  
 Pro Pro Phe Arg Pro Pro Leu Gly Pro Phe Pro Gly Arg Arg  
                           35                          40                          45

<210> 17  
 <211> 60  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:peptide

<400> 17  
 Arg Arg Ile Arg Pro Arg Pro Pro Arg Leu Pro Arg Pro Arg Pro Arg  
           1                          5                          10                          15  
 Pro Leu Pro Phe Pro Arg Pro Gly Pro Arg Pro Ile Pro Arg Pro Leu  
                           20                          25                          30  
 Pro Phe Pro Arg Pro Gly Pro Arg Pro Ile Pro Arg Pro Leu Pro Phe  
                           35                          40                          45  
 Pro Arg Pro Gly Pro Arg Pro Ile Pro Arg Pro Leu  
           50                          55                          60

<210> 18  
 <211> 42  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Description of Artificial Sequence:peptide

<400> 18  
 Arg Arg Arg Pro Arg Pro Pro Tyr Leu Pro Arg Pro Arg Pro Pro Pro  
           1                          5                          10                          15  
 Phe Phe Pro Pro Arg Leu Pro Pro Arg Ile Pro Pro Gly Phe Pro Pro  
                           20                          25                          30  
 Arg Phe Pro Pro Arg Phe Pro Gly Lys Arg  
           35                          40

<210> 19  
<211> 17  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:peptide

<400> 19  
Arg Gly Gly Arg Leu Cys Tyr Cys Arg Arg Arg Phe Cys Ile Cys Val  
1 5 10 15

Gly

<210> 20  
<211> 11  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:peptide

<400> 20  
Leu Phe Glu Ala Ile Glu Gly Phe Ile Phe Leu  
1 5 10

<210> 21  
<211> 10  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:peptide

<400> 21  
Gly Ile Leu Gly Phe Val Phe Thr Leu Thr  
1 5 10

<210> 22  
<211> 14  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence:peptide

<400> 22  
Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu  
1 5 10

<210> 23  
<211> 8  
<212> PRT  
<213> Artificial Sequence



&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence:peptide

&lt;400&gt; 23

Val Tyr Asp Phe Phe Val Trp Leu

1

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